Characterization and Functional Analysis of the Human Inducible Nitric Oxide Synthase Gene Promoter

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ABSTRACT

Background: Nitric oxide has a wide variety of homeostatic and pathological effects. Control of the production of nitric oxide by the inducible form of the enzyme resides in the 5′ promoter region of the gene. Although control of the murine isoform has been investigated, little is known about the functional aspects of the human analog.

Materials and Methods: A 3.9-kb 5′ nontranslated region of the human gene was cloned, sequenced, and several reporter constructs prepared. The promoter-reporter constructs were transfected into human or murine monocytoid cells and reporter expression quantified following cytokine activation of the cells. The production of nitric oxide was also monitored.

Results: Although a murine promoter-reporter functioned efficiently in both human and mouse cells, the human constructs functioned only in human cells. The activity of the mouse construct increased progressively with the addition of activating cytokines, but the human promoter-reporter did not. Although interleukin 1β drove expression of the human inducible nitric oxide synthase reporter, actual expression of nitric oxide required both interleukin 1β and interferon-γ.

Conclusions: The data indicate that despite the significant homology between the human and mouse inducible nitric oxide synthase promoter sequence, control of the two genes is quite different. In addition to being more efficient in promoter activity, the murine promoter responds increasingly to cytokines that are not effective for the human analog. It is also apparent that human inducible nitric oxide synthase is controlled at both the level of transcription and post-translationally.

INTRODUCTION

The free radical nitric oxide (NO) has pleiotropic effects in many mammalian systems, including a central role in controlling blood pressure (1), as a neurotransmitter (2), as an effector in retinotectal innervation (3), and as a microbicidal/tumoricidal factor within some cells of the immune axis (4). More recent data strongly implicate NO as a mediator of tissue damage in a variety of disease states, including autoimmunity and viral infections (5,6), disorders in immune surveillance (7), as well as septic shock and cytokine-induced hypotension (8,9).

Three classes of NO-producing enzymes were initially identified based on their cellular origin or on the mechanism controlling their expression. The distinguishing criteria are the dependence or independence of the enzymes on calcium concentration and the cell type in which the isoform was first identified. The constitutive nitric oxide synthases (cNOS), which include two members first isolated from endothelial cells and neurons, share an expression control mechanism based on intracellular calcium concentration and calmodulin binding (reviewed in Ref. 10); the activity is modulated post-transcriptionally. A third isoform, inducible nitric oxide synthase (iNOS) (11–13) is found in many cell types. It is distinguished by its induction at the transcriptional level by certain bacterial products and inflammatory cytokines via the promoter response elements in the 5′ noncoding region of the gene (reviewed in Ref. 14). Although iNOS also binds calmodulin, it does so at physiological calcium levels present in the resting cell, and thus is not modulated in response to calcium

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fluxes, but rather in response to promoter activation following signal transduction (14).

iNOS is further distinguished from cNOS enzymes in that it appears to be involved primarily as a part of host defense mechanisms. The gram-negative bacterial toxin lipopolysaccharide (LPS) induces murine iNOS expression in several cell types, and iNOS is up-regulated by several cytokines in a species-dependent manner. iNOS is induced in murine macrophages by the synergistic action of LPS and interferon-γ (IFNγ) (14,15). The molecular basis of this synergy was recently elucidated by Xie and coworkers (16), who demonstrated that a 1749-bp segment 5′ to the murine iNOS gene contained a series of 22 regulatory elements. However, only two of the elements were required for up-regulation of a reporter construct mimicking the behavior of native iNOS, but the two elements conferred sensitivity to LPS and IFNγ in a synergistic manner.

More recently, a molecular clone containing the human iNOS 5′ promoter region was obtained (17) and found to be highly homologous to the murine sequence. Functional analysis to determine whether the activation signals effective in inducing expression of mouse iNOS also operate for the human analog were not reported. Intact human iNOS-producing cells have a requirement for at least three concomitant activators for iNOS induction. Certain human cell types, notably macrophages, appear to be completely refractory to iNOS induction, although the murine analogs express high levels of iNOS following cytokine induction.

Because the cloned fragment of the human and mouse iNOS promoters are so similar, we reasoned that perhaps critical promoter response elements may reside 5′ to the previously studied region of the reported sequence. We also noted that the frequency of promoter binding sequences in the human iNOS 5′ region did not diminish toward the 5′ boundary of the reported clone as would be expected if the entire promoter region had been isolated. We therefore undertook the isolation and functional characterization of a significantly longer section of the putative human iNOS promoter region. We report here that an additional 2713-base segment which we have cloned and sequenced contains a high frequency of recognized consensus regulatory elements. Functional studies showed that the critical control elements appear to be clustered within the first 1500-bp region 5′ to the transcription start site. Our studies also indicate that although the human and mouse promoter regions appear homologous based on sequence comparisons, the two regions differ significantly in their ability to up-regulate the transcription of reporter constructs in cells of murine or human origin. Finally, our studies showed that activation of the human promoter-reporter occurred with either interleukin 1β (IL-1β) or tumor necrosis factor α (TNFα) alone, but that IFNγ was absolutely required for the production of NO, indicating that control of NO expression in human cells is controlled at a minimum of two levels.

MATERIALS AND METHODS

Preparation of cDNA Probes

Colonies containing full or partial DNA fragments of the human iNOS 5′-flanking sequence were identified by hybridization with a 425-base fragment obtained by polymerase chain reaction (PCR) from human DNA (12). The fragment was generated by PCR amplification using 5′-AGCT TCCTGGACTCCTGTCA as primer for the region −425 to −406 and 5′-GAACACACTGGCAGC CAAG as reverse primer for the region −19 to −1. Primers were synthesized on an Applied Biosystems 394 DNA synthesizer. Amplified cDNA was sequenced directly on an Applied Biosystems automated 373A sequencer using DyeDeoxy Terminator cycle sequencing with the manufacturer's kit. The probe was radiolabeled using a DNA random priming labeling kit according to the manufacturer's recommendations (Boehringer-Mannheim, Indianapolis, IN, U.S.A.).

Isolation of Genomic DNA Clones for the Promoter Region

A human genomic library in lambda phage derived from placenta (Clonetech, La Jolla, CA, U.S.A.) was diluted and plated. Approximately 5×10^5 plaques were screened using a ^{33}P-labeled cDNA fragment generated by PCR. Two colonies specifically hybridized with the probe and were picked for further characterization. Based on preliminary experiments, the region of interest lay between an *Xba*1 and an *Eco*R1 site within the clones. The colonies were digested with the enzymes and the products separated on a 1.0% agarose gel. The restriction fragment identified by southern hybridization was directly cloned into a pBluescript vector (Stratagene, San Diego, CA, U.S.A.) and sequenced using an automated DNA sequencer. The murine iNOS pro-

moter region was prepared by PCR using a 5′ primer, AAAAACGTACACGAGGTGCVGACT and a 3′ primer AAAACTGAGGTGATCTACTCCG based on the previously published sequence (16). The PCR fragment was digested with *Mlu*I and *Xho*1, and inserted in the PGL Basic vector upstream of the luciferase gene (Promega). The human 3.9-kb clone was digested with *Kpn* 1, and the resulting 3.8-kb fragment was ligated into PGL, yielding a plasmid with a total size of 9300 bp. The 3.8-kb fragment was also digested with *Pst* 1 to yield a 1.8-kb fragment which was cloned into PGL, yielding a plasmid with a size of 7500 bp and generating a construct similar to the murine 1.5-kb promoter/reporter. The 425-bp promoter/reporter construct was produced by ligating the PCR product described above into PGL.

Cell Culture and Transient Transfections

RAW 264.7 murine macrophages and A549 human epithelioid cells derived from a lung adenocarcinoma were obtained from ATCC (Rockville, MD, U.S.A.). GO-G-UVW astrocytoma cells were obtained from the European Collection of Animal Cell Culture (Salisbury, United Kingdom). RAW 264.7 cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 50 μ/ml penicillin, 50 μg/ml streptomycin and 5 μM L-glutamine, A549 cells were cultured in Hams F-12 medium with 10% heat-inactivated fetal calf serum, 50 μg/ml streptomycin, 50 μ/ml penicillin, and 5 μM L-glutamine.

Cells were activated for NO production using a modification of a previously published protocol (18). TNFα was purchased from Sigma (St. Louis, MO, U.S.A.) and diluted to the indicated concentration. IL-1β and IFNγ (Sigma) were also added to cultures as indicated. Neutralizing antibodies to IL-1β and IFNγ were purchased from Biosource International (Camarillo, CA, U.S.A.) and mixed at a 10-fold excess with the appropriate cytokine for 1 hr prior to addition of cytokines to cultures.

Transfections were performed with Lipofectin according to the manufacture's (Gibco/BRL, Gaithersburg, MD, U.S.A.) recommendations in 6-well plates. The culture medium was completely replaced 24 hr after transfection, and new medium containing replenishment cytokines added as indicated. After an additional 8–24 hr, the cell monolayer was washed twice with PBS, and the cells were lysed by the addition of 250 μl of reporter lysis buffer (Promega). An aliquot of the lysate was tested for luciferase activity using a commercial kit (Promega Luciferase Assay System) and the results quantitated with a scintillation counter.

Quantitation of NO Production

Aliquots of the cell lysate prepared for luciferase quantitation were incubated with 50 mM HEPES containing 1 mM NADPH, 10 μM (6R)-5,6,7,8-tetrahydro-L biopterin HCL, 100 μM $CaCl_2$, 5 μ/ml calmodulin (Sigma), 1 mM DTT, 10 μM FAD, and 1 mCi [^{3}H]-L-arginine (36.8 Ci/mM; Dupont/New England Nuclear, Cambridge, MA, U.S.A.). Because of the cNOS activity in A549 cells (18), the cNOS inhibitor trifluoperazine (Sigma 100 μM) was added to all NO assays so that the detected activity represented only iNOS. Some experiments were also done in the presence of either 100 μM methyl L-arginine or the enantiomer D-arginine, which was approximately 10-fold less effective in blocking the enzymatic conversation of arginine to citrulline. The mixture was incubated for 60 min at 37°C. A 150-μl aliquot was loaded onto a cation exchange column (AG 50W-Xg; Bio-Rad, Richmond, CA, U.S.A.) and eluted with water. The eluate was collected and the radioactivity quantified by liquid scintillography.

The sequence of the human iNO synthase promoter region reported here was submitted to Genbank, and the accession number is Z49251.

RESULTS

We have cloned and completely sequenced a 3923-bp region of the human iNOS gene which includes the transcriptional start site of the iNOS enzyme as well as the first exon of the gene. This clone contains the 425-bp promoter region previously reported by Chartain and coworkers (12) (GenBank accession number L26055) as well as the additional 675-bp region of the 5′ sequence characterized by Nunokawa et al. (17) (EMBL accession number D29675). Direct comparison of the sequence of this 3923-bp clone with previously reported sequences indicated complete agreement with two minor exceptions: (i) the sequence reported by Nunokawa et al. contained a G at −276, while ours and that of Chartain have an A (Fig. 1); (ii) in the 5′ region distal to that sequenced by Chartain, we found a AAA triplet at −1034 through −1032, which was not reported by Nunokawa (17).

Analysis of the nucleotide sequence of the 5′ noncoding region of the human iNOS gene re-

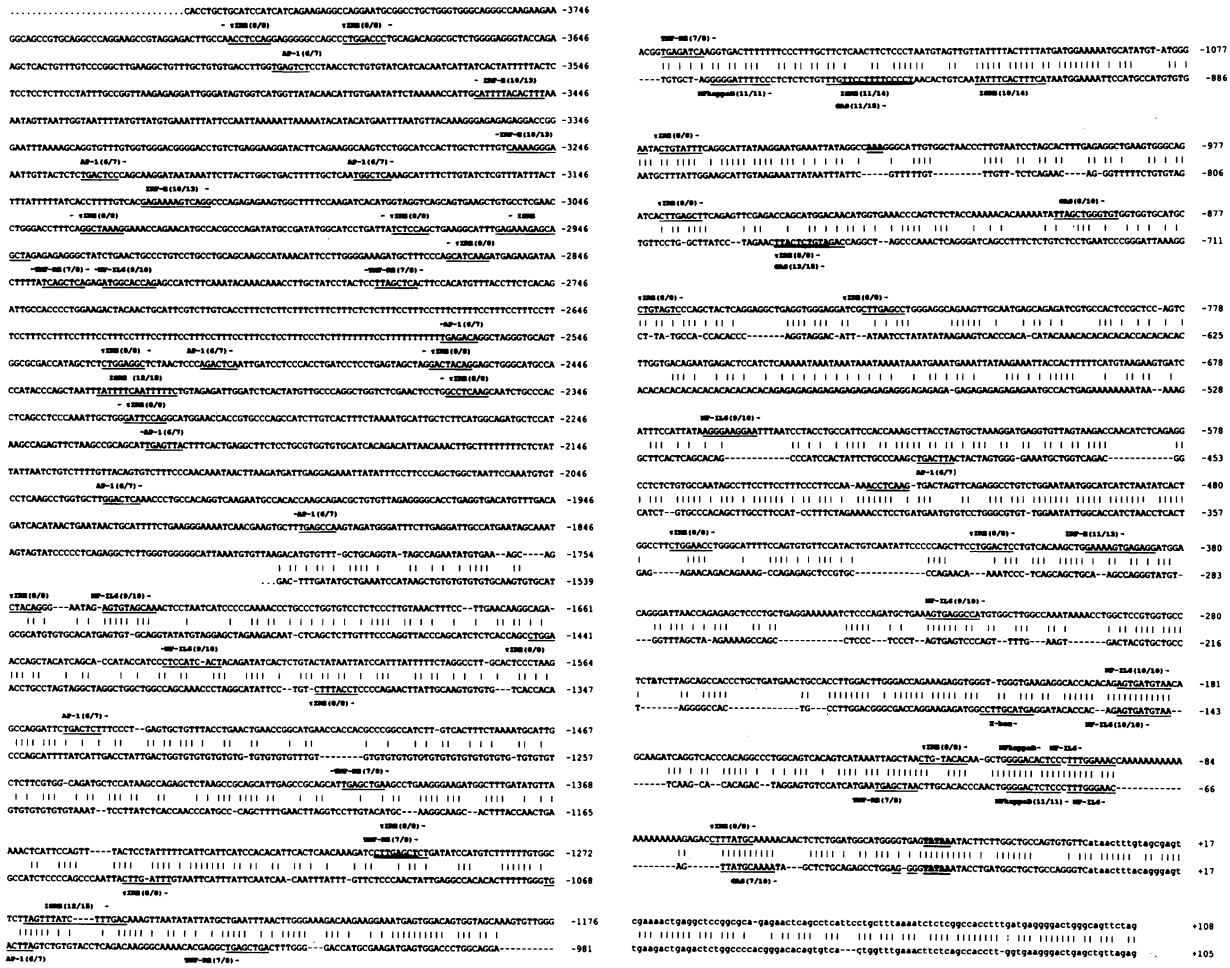

FIG. 1. Comparative sequence of the mouse and human iNOS promoter regions and identification of consensus promoter response elements

The sequence of the entire 3814-bp human iNOS promoter region cloned and sequenced is presented. The previously published murine sequence is presented as the lower series of rows (GenBank accession numbers L23806 and L09126). The transcriptional start site, indicated by lower case letters, was identified from Ref. 12. The differences in the sequences discussed in the text are presented in bold. Promoter response elements were identified using the consensus sequences cited in Table 1, and the position and orientation are identified by arrows under the indicated regions. Only exact matches, or matches as previously reported are shown. Alignment matching of the murine and human sequences was determined by using the NALIGN program of the PCGene package (Intelligenetics, Mountain View, CA, U.S.A.) using the default values for weighting open gaps as 10 and unit gap costs as 10.

vealed consensus sequences for different transcriptional regulatory elements. In addition, two copies of the *Alu*1 sequence element are contained in this DNA at positions −2561 to −2276 and −1030 to −706. The 5′-most repeat is oriented in the opposite direction to the 3′ repeat. Table 1 summarizes the identified promoter response elements (PREs) that had exact matches with consensus sequences or that differed by a limited number of bases. The previously reported murine promoter is included for comparison.

To test the functional integrity of the entire human iNOS promoter region, several reporter constructs were generated containing a luciferase gene 3′ to the *Xba* I site used to isolate the 3800-bp fragment from the human DNA library. A similar construct was made using the murine iNOS promoter region as described by Xie and coworkers (16). The reporter constructs were transfected into the murine macrophage cell line RAW 264.7 or the human lung epithelioid cell line A549 which expresses NO following exposure to specific cytokines (18). As shown in Fig. 2, the mouse promoter efficiently drove the tran-

TABLE 1. Frequency of promoter response elements in the human 3.8-and 1.8-kb and mouse 1.5-kb promoter regions

Element	Consensus Sequence	Ref.	n/n[a]	Human 3.8-kb Clone[b]		Human 1.8-kb Clone[c]		Mouse 1.5-kb Clone[d]	
γ IRE	CTKKANNY[e]	24	8/8	11+	8−	9+	1−	4+	0−
			7/8	141+	140−	50+	52−	5+	1−
AP-1	TGACTCA	25	7/7	0+	0−				
			6/7	5+	4−	3+	1−	1+	1−
NF-IL6	AGTTANGNAA	26	10/10	1+	0−	1+	0−		
			9/10	4+	2−	3+	1−		
NF-κB	GGGRNNYYCC	27	10/10	1+	0−	1+	0−	2+	0−
TNF-RE	TGAGCTCA	28	8/8	0+	0−				
			7/8	5+	n/a	3+	n/a	2+	n/a
X-box	CCYAGMRACNG	24	8/11	0+	0−			1+	0−
GAS	TTACTCTAAA	24	8/10	0+	0−			1+	0−
IRF-E	SYAAAGYSAAA(A)G	29	11/13	1+	0−	0+	0−	0+	0−
			10/13	0+	2−	0+	2−		
ISRE	YAGTTTC(A/T)YTTTCC	30	11/14	2+	2−	1+	2−	2+	0−

The sequence of the 3814-bp iNOS human promoter (H3.8), or the 1800-bp 5′ fragment (H1.8) analogous to the 1588-bp murine iNOS promoter previously reported by Xie et al. (16) was analyzed using a NALIGN program with the PRE consensus sequences listed. The PREs identified bind proteins induced in response to the activating factors LPS, IFNγ, IL-1β or TNFα used in the experiments reported here. The mouse sequence is presented for a more direct comparison of the number of resident PREs.

[a] Base match present in sequence compared with the PRE consensus sequence. Where less than a perfect match is tabulated, the mismatch was also reported to be active as a PRE.

[b] Number of PREs identified in the entire human 3.8-kb iNOS promoter region in either the positive (+) or negative orientation (−).

[c] Number of PREs identified in the entire human 1.8-kb iNOS promoter region in either the positive (+) or negative orientation (−).

[d] Number of PREs identified in the mouse 1.5-kb iNOS promoter region in either the positive (+) or negative orientation (−) derived from a previously published analysis.

[e] Consensus sequence of PRE.

scription and translation of the reporter gene in murine cells whereas the human promoter failed to express the luciferase reporter in the RAW 264.7 mouse macrophages. In contrast, activated A549 cells efficiently expressed increased luciferase activity following transfection with either the human or mouse promoter. Cotransfection of either the human iNOS promoter-reporter or the murine analog with a β-galactosidase control plasmid (Promega) yielded similar activities, indicating that the efficiency of transfection was similar in both cell types. Briefly, cells were transfected simultaneously with one of the iNOS promoter-reporter constructs and equal concentration of the control plasmid. Following cytokine activation, the concentrations of both luciferase and β-galactosidase products were determined. Luciferase activity was normalized to the activity of the β-galactosidase transfection control.

We examined the activity of the murine and human reporter plasmids more extensively in A549 cells. Three different lengths of the human promoter were tested to identify the critical regions responsible for biological activity (Fig. 3). The 425 bp proximal to the transcription start site had minimal to no promoter activity. The 1800-bp clone, which closely matched the murine construct in size and original genomic position, had a significantly greater promoter activity, while the entire 3700-bp promoter region demonstrated less activity. The activity of the murine promoter-reporter construct consistently exceeded that of the human analog by 5- to 7-fold in A549 cells (Fig. 3, inset). The differences in observed activity could not be attributed to differences in transfection efficiencies, based on the relative activity of the cotransfected β-galactosidase expression control construct (data not

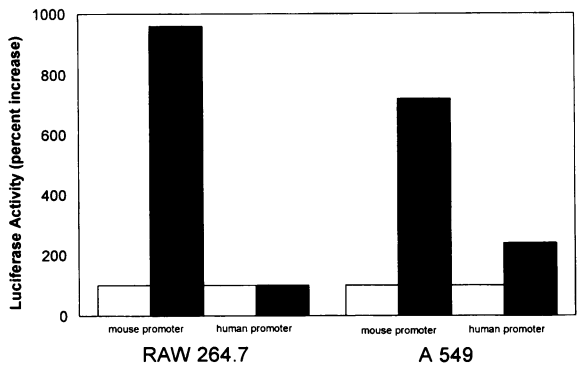

FIG. 2. Murine and human iNOS promoter activity in both human and mouse cells

RAW 267.4, and A549 cells were cultured as described. Transfections were performed as described in Materials and Methods. Equal numbers of cells (1×10^6) were used in all cases. Luciferase activity is presented as percent increase over that present in nonactivated cells. RAW 267.4 cells were stimulated with LPS (10 μg/ml, solid bar), or nonstimulated (open bar). A549 were transfected with promoter/reporter constructs, incubated for 24 hr and then activated with IL-1β (100 μ/ml), IFNγ (500 μ/ml), and TNFα (10 ng/ml) for 8 hr (solid bars) or nonstimulated (open bars).

shown). Additionally, although the 1800- and 3700-bp constructs differed significantly in size, incorporation into PGL vectors resulted in more similar total masses (7500 versus 9300 bp) minimizing the molar differences used in transfection.

Because the 1800-bp promoter-reporter construct showed maximal activity in our test system, we used it for a comparison of the relative influence of the cytokines present in the activation mixture with both mouse and human promoter-reporter constructs. With a mixture of IL-1β, IFNγ, and TNFα, the promoter activation reached a maximum at 8 hr (Fig. 3, inset) and this was consequently used as the standard activation period in all subsequent experiments. A549 cells were transfected with the human or mouse constructs and then exposed to an 8-hr cytokine activation period. The product of the reporter gene was then quantified, and the results are presented in Fig. 4. The human promoter driving luciferase expression had the greatest activity when cells were treated with IL-1β alone, and this maximum was slightly but significantly diminished in the presence of IFNγ. TNFα used alone resulted in slight stimulation of the human iNOS promoter, while IFNγ alone had no stimulatory activity for the promoter-reporter construct in A549 cells. The activity of the mouse promoter increased with addition of IFNγ and TNFα along with IL-1β, while the activation of the human promoter diminished when TNFα or IFNγ was added to IL-1β. For the mouse promoter, the greatest stimulation was achieved with the combination of IFNγ, TNFα, and IL-1β. Thus, combined effects of IL-1β with other cytokines differed for the human and mouse promoters tested in A549 cells. The specificity of the cytokines stimulating effect was demonstrated by the absence of induced activity when neutralizing antibodies to the respective cytokines were added before cytokine stimulation (data not shown). LPS had no detectable effect on the induction of the promoter as measured by luciferase activity (not shown).

Simultaneous analysis of both luciferase and iNOS activity in A549 cells transfected with the human 1800-bp promoter/reporter construct following stimulation with one or more cytokines revealed differences in the relative stimulation of the two activities (Fig. 5). Activation of luciferase was evident in A549 cells treated with either IL-1β alone or, to a lesser extent, TNFα alone, whereas only a small increase above baseline was seen in iNOS activity under either of these conditions. The single addition of IFNγ had a marginally suppressive effect on both luciferase and iNOS activities. IL-1β, the most potent upregulator of reporter gene activity in A549 cells, did not significantly increase the production of NO. However, addition of IFNγ together with IL-1β greatly enhanced the production of NO over that seen with IL-1β, while slightly but consistently decreasing the measured luciferase product. When TNFα was added together with IL-1β and IFNγ, an even larger increase in NO production was observed, while the activity of the luciferase reporter was unaffected or suppressed. Nitric oxide production, as determined by the arginine to citrulline conversion assay were confirmed by reverse transcriptase PCR and Northern blot assays detecting iNOS-specific mRNA, and nitrite concentrations in culture supernatants were quantified by the Griess reaction (data not shown).

DISCUSSION

We have isolated, cloned, and sequenced a 3814-bp region of the human iNOS promoter region which contains an additional 2713 bp beyond that previously published (12,17). We

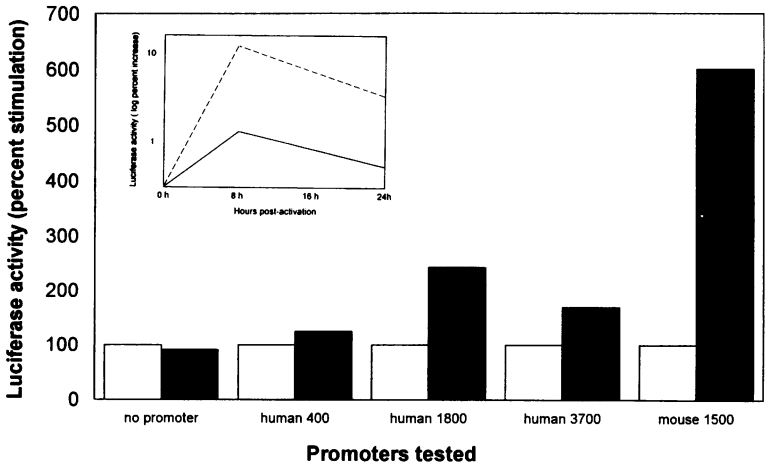

FIG. 3. Comparative activity of human iNOS promoter of different length in A549 cells

The activity of different length constructs of human iNOS promoters coupled to a luciferase reporter gene was measured following transfection and cytokine activation as described in Materials and Methods. Results from nonactivated cells are presented as open bars and cytokine-activated A549 cells as solid bars. A 1500-bp murine promoter was also tested. Equal numbers of cells were transfected with the constructs and luciferase activity was quantified after 8 hr of cytokine treatment. Inset shows the time course of luciferase activity in activated A549 cells with both the human and murine iNOS promoters. The luciferase activity was quantified at 8 and 24 hr postactivation and data are presented as the percentage increase (log scale) over that found in nonactivated cells. Activity of both the human (solid line) and the murine (dotted line) reporter constructs was maximal at 8 hr after cytokine activation of the cells.

found that the entire control region contains sites with homology to recognized PREs, although the density diminishes toward the 5′ end of the 3814-bp clone described here. Previous work by others has provided data that localize the PRE sites with the greatest activity in the induction of the mouse iNOS gene. To date, no work has been published on similar sites within the human promoter region. Within the murine iNOS promoter, two regions were found to be critical for the induction of iNOS by LPS and for the synergistic effect of IFNγ (19). LPS induction requires an element within the first 209 bases 5′ to the transcriptional start site, while the effect of IFNγ appears to be mediated by elements present in the 5′-most half of the promoter. Comparison of the sequence derived in our studies for the human iNOS promoter with that of the murine promoter indicates extensive nucleotide and structural homology as well as organizational similarities presence and location of specific response elements.

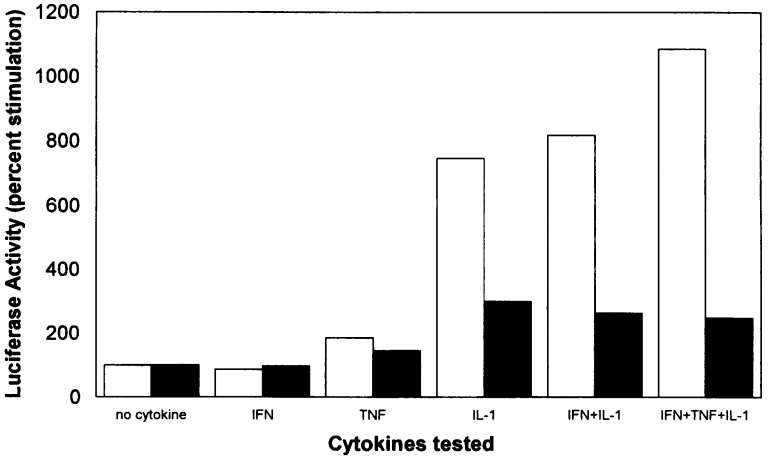

FIG. 4. Activation of the mouse 1.5-kb and human 1.8-kb promoter regions transfected into human A549 cells stimulated with single or multiple cytokines

Luciferase activity was quantified after the transfection of either the murine 1.5-kb promoter/reporter construct (open bars) or the human analog (solid bars) into equal numbers of A549 cells followed by 8 hr of activation with the indicated cytokines (100 μ/ml IL-1β, 10 ng/ml TNFα, or 500 μ/ml of IFNγ alone, or in combinations as indicated). Data are presented as the percentage increase over that present in nonactivated cells.

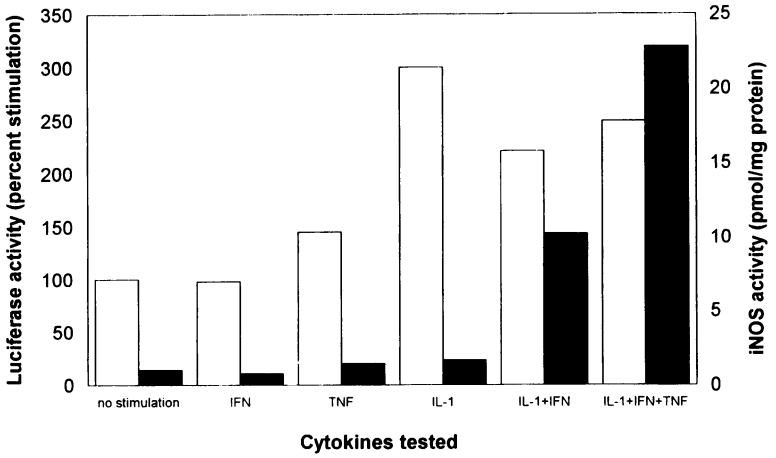

FIG. 5. Comparison of the luciferase activity using the human 1800-bp promoter and nitric oxide production of A549 cells activated with various cytokines alone or in combination

Equal numbers of A549 cells were transfected with the human 1800-bp reporter construct followed by cytokine activation. Aliquots of the transfected cells were collected 8 hr after activation and analyzed for both luciferase activity (open bars) and NO production by the arginine to citrulline assay (solid bars). Cytokines were added singly or in combination using 100 μ/ml IL-1β, 10 ng/ml of TNFα, or 500 μ/ml of IFNγ. Luciferase activity is presented as the percentage increase over that seen in nonactivated cells. Nitric oxide production is presented as picomole of [^{3}H]-citrulline produced per milligram of cellular protein. Culture supernatant concentrations of nitrite were determined by the Griess reaction and found to correlate with that determined by the arginine to citrulline assay (data not shown).

To determine the regions of the promoter region necessary for the activation of human iNOS, we constructed three reporter plasmids containing a luciferase reporter gene coupled to 400-, 1800-, or 3800-bp fragments of the cloned human promoter as well as a construct containing a 1500-bp fragment of the mouse iNOS promoter region previously reported (16). Transfection experiments showed that the 1800-bp promoter-reporter construct was the most active in human A549 cells. The 400-bp promoter construct demonstrated minimal to no activity, perhaps because the region contained the PREs previously found to be important only for LPS induction (16,19). We found that LPS had no effect on promoter induction in A549 cells, probably due to the absence of the CD14 cellular receptor required for endotoxin inducibility (20). Interestingly, the 3800-bp human promoter/reporter construct was less potent than the 1800-bp fragment. Analysis of the cloned sequence upstream of the 1800-bp fragment failed to identify any known negative regulatory elements, raising the possibility that a novel element of this region may negatively affect transcription of the gene. Alternatively, the presence of oligomeric repeats of AT as well as two Alu repeats in this region of the human promoter structure, which are features consistent with a gene duplication event, may have interrupted the integrity of the human relative to the murine iNOS promoter.

No stimulation of the human promoters was observed in murine RAW 247.8 cells, although the murine promoter was highly activated. The lack of activity of any of the human-derived constructs in mouse cells suggests that despite the numerous similarities between the mouse and human promoter sequences, specific factors required by the human promoter are missing from the murine environment, or are not active with the human promoters. Interestingly, experiments showed that the human hepatoma Hep-G2 cell line, which did not produce NO in response to cytokine stimulation and also failed to support the activation of the human 1800-bp promoter/reporter construct, did support the expression of the murine analog (data not shown). The murine construct functioned well in both human and mouse cells, and the activity of the construct was consistently greater than that of its human counterpart in all cells tested (A549, GO-G-UVW astrocytoma, HepG2, and RAW cells).

Comparison of the ability of the 1800-bp human promoter construct to drive the transcription of the luciferase gene with the concomitant production of NO from the endogenous

human iNOS promoter revealed discordance in the relative stimulation by different cytokines (Fig. 5). IL-1β activation alone was sufficient to significantly upregulate transcription of the luciferase reporter gene, but no concomitant increase in NO production was observed. With the addition of IFNγ, NO production, but not luciferase activity, was significantly increased. Because addition of IFNγ did not induce increased NO production when tested alone, it must complement the stimulatory activity of IL-1β in an indirect manner. TNFα, when added to IL-1β plus IFNγ further increased NO production, but did not increase luciferase activity suggesting that the mechanism of action of TNFα is different from that of IFNγ; the latter two cytokines acted in an additive manner for the increased expression of NO, but not for the human iNOS promoter driven reporter construct. We found no evidence of this for the increase in the expression of the reporter luciferase gene (Fig. 5).

Previous investigators have reported the effects of various cytokines, including IFNγ, on NO inducibility by assaying the increase in iNOS mRNA levels (21,22). Levels of mRNA were unaffected by IFNγ, suggesting that its activity in up-regulating NO production is exerted after transcription. Interferons were shown to stabilize mRNAs containing octomer repeats of UUAUUUAU in the 3′ untranslated regions of certain cytokine-induced mRNAs (23), and iNOS mRNA stability is reportedly governed by the same mechanism (21). The human iNOS gene reported by Chartain et al. (12) contains such repeats in the 3′ untranslated region, but the luciferase gene used in the present experiments did not. This suggests one possible mechanism to explain the ability of IL-1β to drive human iNOS promoter luciferase activity, but not to up-regulate the expression of NO. IFNγ might either stabilize the iNOS mRNA or selectively prevent the synthesis of a protein critical to the iNOS mRNA destabilization process. Alternatively, other regulatory elements might be within the iNOS gene itself or further downstream. Experiments to test these possibilities are in progress.

The experiments reported here indicate that, although the human and murine iNOS genes share significant sequence and structural homology, differences exist in the control of their respective requirements for activation. The data also suggest that human iNOS expression is influenced by both transcriptional and post-transcriptional events, as expected for a gene with such a broad homeostatic role and pathogenic potential. Further work is needed to understand the full range of mechanism controlling iNOS expression in the human cell types that express iNOS.

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